Supplementary materials

Table 1: Antibodies used in this study

Antibodies			
Ab name	**Company**	**Identifier**	**Dilution**
Anti-β-tubulin	Sigma Aldrich	T5201	1:1000 (IB)
Anti-GAPDH (14C10)	Cell signaling technologies	2118s	1:2000 (IB)
Anti-FLAG® M2	Sigma Aldrich	F1804	1:1500 (IB) 3 µg/mg lysate (IP)
Streptavidin-HRP	Jackson Immunoresearch	16-030-084	1:500 (IB)
Anti-MAP4K4	Abcam	ab80418	1:2000 (IB)
Anti-STRN4	Abcam	ab194948	1:2000 (IB)
Anti-STRN3 (S68)	Thermo Fisher Scientific	MA1-46461	1:2000 (IB)
Anti-STRIP1	Abcam	ab199851	1:2000 (IB)
Anti-VASP (9A2)	Cell signaling technologies	3132	1:1000 (IB) 1: 400 (IFA)
Anti-phospho-VASP (Ser157)	Abcam	ab47268	1:750 (IB)
Anti-mouse HRP linked	Cell signaling technologies	7076	1:5000 (IB)
Anti-rabbit HRP linked	Cell signaling technologies	7074	1:5000 (IB)
easyBlot anti-mouse HRP	GeneTex	GTX221667-01	1:1000 (IB)
Anti-BioID2	Novus	NBP2-59940	1:200 (IFA)
Streptavidin Alexa Fluor 594 conjugate	Invitrogen	S11227	1:500 (IFA)
Anti-Calbindin	Abcam	ab108404	1:1000 (IFA)
Anti-GFAP	Abcam	ab53554	1:250 (IFA)
Anti-human nuclei (3E1.3)	Millipore	MAB4383	1:250 (IFA)
Donkey anti-Mouse IgG Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A32766	1:400 (IFA)

Cy3-conjugated Donkey anti-Rabbit IgG	Jackson Immunoresearch	711-165-152	1:250 (IFA)
Brilliant Violet 421 Donkey Anti-Goat IgG	Jackson Immunoresearch	705-675-147	1:100 (IFA)
Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor 405	Thermo Fisher Scientific	A31553	1:200 (IFA)
Hoechst	Sigma Aldrich	B2883	1:2000

Table 2: Sequence information for sgRNAs used in this study

sgRNA Oligonucleotides		
Name	**Exon**	**Target sequence**
sgCTRL	-	GTAGCGAACGTGTCCGGCC
sgSTRN4#1	Exon 1	GCTCAGGTGGCCTTCCTTCA
sgSTRN4#2	Exon 2	TTCCTTCAGGGAGAGAGGAA
sgSTRN3#1	Exon 2	AGGTCAAGAGAACCTGAAGA
sgSTRN3#2	Exon 2	AGTATGCATTAAAACAAGAA
sgSTRIP1#1	Exon 4	TGCCAGGGAGAAGAGACTCA
sgSTRIP1#2	Exon 4	GGATGGCTTGGAAGTCACTG
sgMAP4K4#1	Exon 7	GGGCGGAGAAATACGTTCAT
sgMAP4K4#2	Exon 4	CAGGACATGATGACCAACTC

Table 3: siRNA sequences used in this study

siRNA			
Name	**Target sequence**	**Company**	**Identifier**
siCTRL	UAAGGCUAUGAAGAGAUAC	Dharmacon	D-001210-02-05
siSTRN4	GGAUCAAGAUGCUAGAGUA	Dharmacon	D-020389-01-0002
siSTRN3	GGAGGAGGCAAGUCAUUUA	Dharmacon	D-019145-01-0002
siSTRIP1	GCAGCAAAUUUAUAGGUUA	Dharmacon	D-021516-01-0002

siMAP4K4	UAAGUUACGUGUCUACUAU	Dharmacon	D-003971-05-0002

Table 4: Custom qPCR primers used in this study

qPCR primers			
Target genes		**Sequence**	**Product size**
18s	F	GGATGTAAAGGATGGAAAATACA	23 bp
	R	TCCAGGTCTTCACGGAGCTTGTT	
MAP4K4	F	GTTACACTAATGCGCACCAC	193 bp
	R	GTACTTGCCACCAGTCTGCT	
STRN4	F	CTCAGGTGGCCTTCCTTCAG	20 bp
	R	TTTGGCCCTTTCCTGCTTCA	
STRN3	F	TGGCACAGAATGGGCTGAAC	101 bp
	R	CTCCAAGGCCCAGTACACTT	
STRIP1	F	CGCAAAGACTCAGAGGGCTA	109 bp
	R	GCCCTTCCGTGTAGCTGTAA	
CTGF	F	CACCCGGGTTACCAATGACA	119 bp
	R	GGATGCACTTTTTGCCCTTCTTA	
CYR61	F	ACAGCAGCCTGAAAAAGGGC	104 bp
	R	GGGCCGGTATTTCTTCACACT	
ANKRD1	F	TAGCGCCCGAGATAAGTTGC	97 bp
	R	GTCTGCCTCACAGGCGATAA	

Supplementary figures

Figure S1: BioID identified MAP4K4 interactome. (a) Schematic diagram of BioID technology and schematic representation of the lentiviral vectors used to generate 3xFLAG-tagged MAP4K4-BioID2 cell lines. Biotin ligase (BioID2) was fused to either the N-terminus (FLAG-BioID2-MAP4K4) or C-terminus (MAP4K4-BioID2-FLAG) of MAP4K4. An extended flexible linker consisting of 13 repeats of GGGGS was inserted between the coding regions of MAP4K4 and BioID2. As negative control, BioID2-FLAG was used (adapted from[82]). **(b)** Confocal microscopy images of DAOY cells expressing BioID2-MAP4K4 fusion protein or BioID2 alone. Biotinylated proteins were labeled with streptavidin (red) and predominantly co-localized with BioID2 (green). Considerable biotinylation is observed in biotin (50 µM) supplemented cells expressing BioID2. DNA is labeled with Hoechst (blue). Scale bar: 30 µm. **(c)** Volcano plot of protein interactions identified by streptavidin affinity purification-MS in N-BioID2-MAP4K4 (top) or C-BioID2-MAP4K4 (lower) DAOY cells compared to BioID2-CTRL. The logarithmic ratios of protein intensities are plotted against negative logarithmic p-values of Student's *t*-test. *n*=2. Proteins significantly enriched ($p < 0.05$ and > 2-fold enrichment) in BioID2-MAP4K4 cells compared to BioID2-CTRL are indicated in red. Members of the STRIPAK complex are highlighted. **(d)** DAOY cells expressing BioID2-MAP4K4 fusion protein were serum-starved overnight and treated with 1 µM GNE-495 for 16 h where indicated. FLAG-immunoprecipitated MAP4K4 was subjected to immunoblot analysis for STRIPAK components. GAPDH was used as loading control.

Figure S2: STRIPAK complex members are highly expressed in MB patients. (a, b) mRNA **(a)** and protein **(b)** levels of STRN4, STRN3, and STRIP1 in a cohort of 218 pediatric brain tumor samples representing seven histological types, including medulloblastoma (MB, *n*=22), low-grade glioma (LGG, *n*=93), high-grade glioma (HGG, *n*=25), ependymoma (EP, *n*=32), craniopharyngioma (CP, *n*=18), ganglioglioma (GG, *n*=18), and atypical teratoid rhabdoid tumor (ATRT, *n*=12). The graphs represent the Z-score values for protein and mRNA in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA). **(c)** Heatmap for protein or phosphoprotein levels of the indicated proteins in the same cohort of patients as in **a**. **(d)** Scatterplot showing the phospho-abundance of the indicated residues of MAP4K4 versus the protein expression levels of STRN4, STRN3, or STRIP1 in MB samples. The data were obtained from the CPTAC data portal (http://pbt.cptac-data-view.org/).

Figure S3: CRISPR/Cas9 and siRNA-mediated downregulation of MAP4K4 and STRIPAK complex members. (a, b) Immunoblots showing CRISPR/Cas9-mediated depletion of MAP4K4, STRN4, STRN3, or STRIP1 in DAOY **(a)** or HD-MBO3 **(b)** cells. The numbers indicate the sgRNA used for each gene. **(c)** Spheroid invasion assay (SIA) of DAOY cells with CRISPR/Cas9-mediated knockout of MAP4K4, STRN4, STRN3, or STRIP1 with or without stimulation with EGF (30 ng/ml). *n*=3, means ± SD. **(d)** qRT-PCR analysis of MAP4K4, STRN4, STRN3, and STRIP1 in DAOY cells 72 h after transfection with the indicated single siRNA or siRNA combination. *n*=3, means ± SD. **(e)** Immunoblot analysis of MAP4K4 and STRIPAK members in DAOY cells 72 h after siRNA transfection. **(f)** SIA of DAOY cells stimulated with 30 ng/ml EGF and treated with different concentrations of LB-100. *n*=3, means ± SD. **(g)** CellTox Green viability analysis of DAOY spheroids treated for 24, 48, and 72 h with increasing concentrations of LB-100. *n*=3, means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA).

Figure S4: High-resolution images of organotypic cerebellum slice culture implanted with DAOY spheroids. Confocal microscopy analysis of OCSCs co-cultured with siRNA-transfected DAOY tumor cell spheroids 72 h after transfection. 63x image acquisition of representative slices 48 h after implantation without GF treatment. Green: Lifeact-EGFP; blue: calbindin (Purkinje cells); red: GFAP; yellow: Edu-Click-IT. Scale bar: 50 µm.

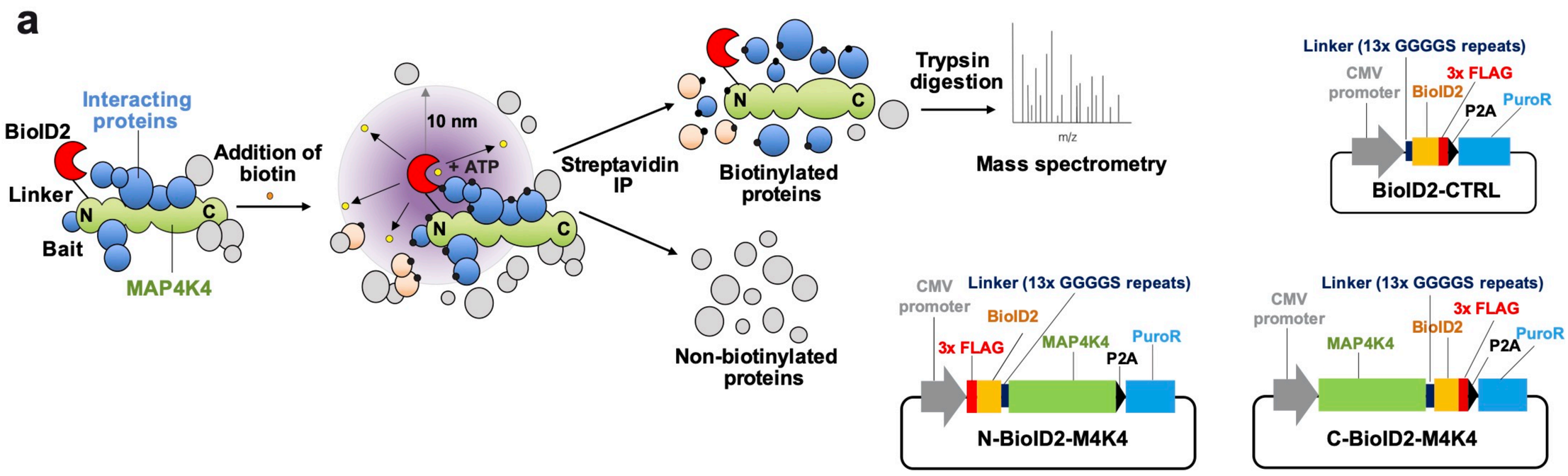
Figure S5: High-resolution images of organotypic cerebellum slice culture implanted with HD-MBO3 spheroids. Single confocal microscopy sections of OCSCs implanted with tumor spheroids derived from HD-MBO3 with CRISPR/Cas9-mediated knockout of MAP4K4, STRN3, or STRIP1. 63x image acquisition of slices five days after implantation and treatment with 12.5 ng/ml bFGF. 4x magnifications of boxed areas are shown. Green: Lifeact-EGFP; blue: calbindin (Purkinje cells); red: human nuclei; yellow: Edu-Click-IT. Scale bar: 200 µm.

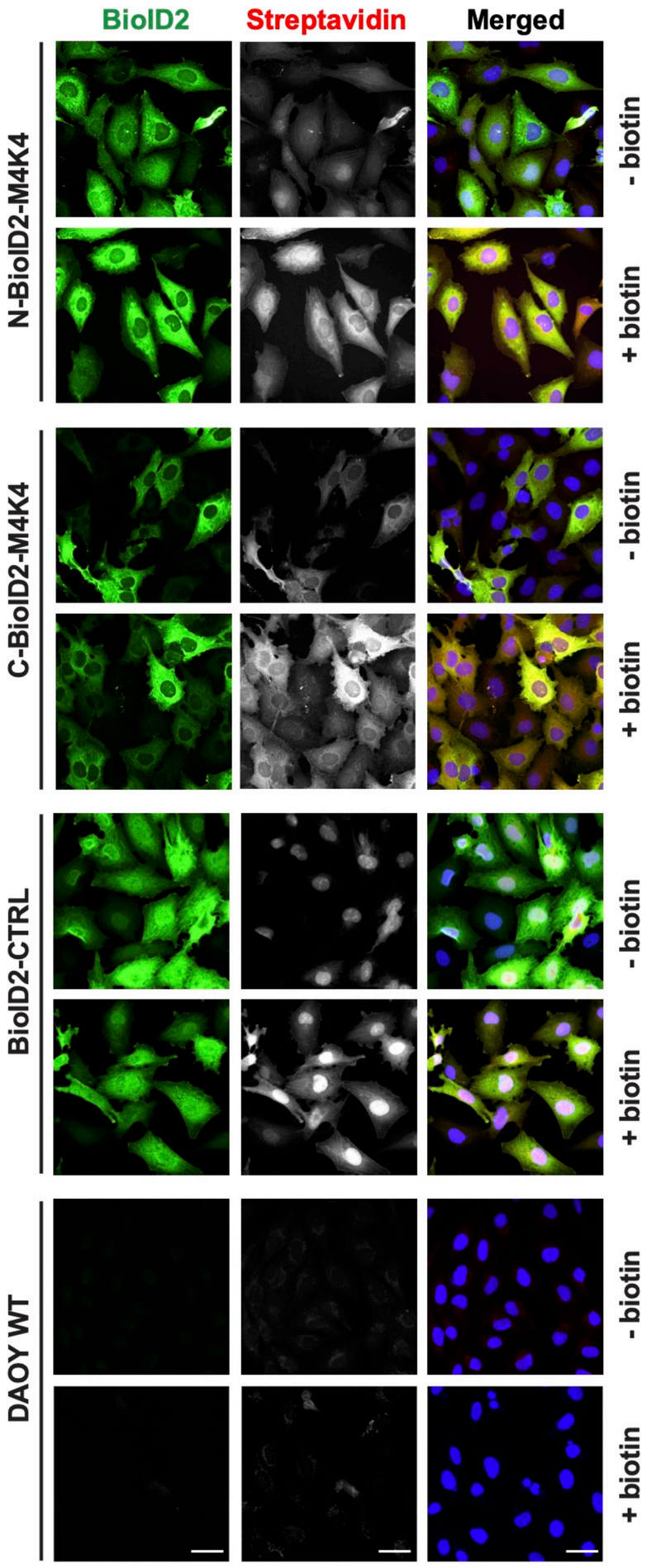
Figure S6: Models for MAP4K4 and STRIPAK regulation of YAP/TAZ target gene expression. MAP4K4 promotes LATS1/2-mediated activation of the Hippo pathway, causing YAP degradation[37]. STRIPAK enables PP2A-mediated dephosphorylation of MAP4K4 and therefore acts as a negative regulator of the Hippo pathway, activating a YAP transcriptional program and ultimately promoting cell proliferation.

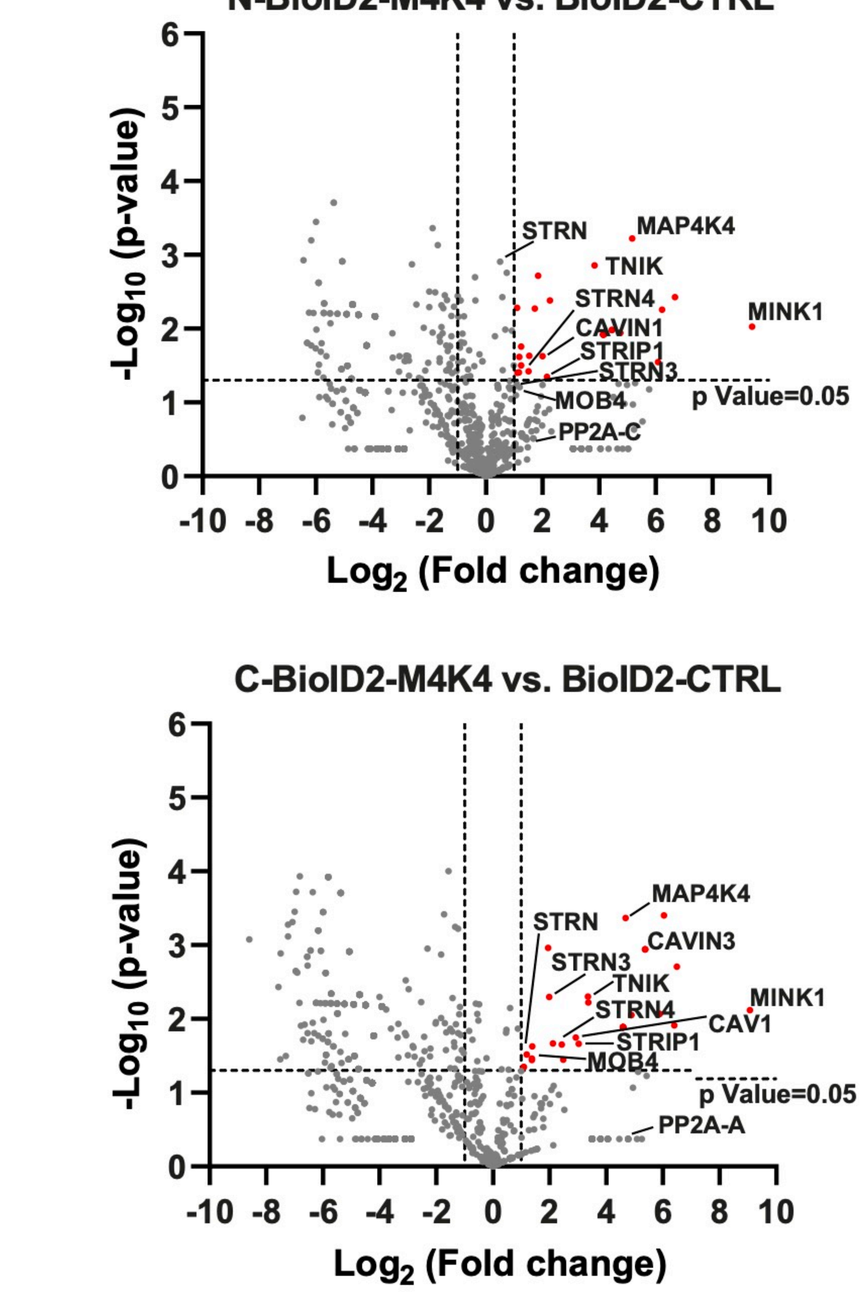
Figure S7: Phylogenetic distribution of the kinases predicted the peptide chip array. **(a)** Upstream kinase prediction analysis of DAOY cells transfected with the indicated siRNA ± treatment with 100 ng/ml bFGF. The plots show the top differentially activated putative upstream tyrosine kinases (PTK) predicted to phosphorylate the phosphosites on the PamChip®. The x-axis indicates the values for the mean kinase statistic, which represents the difference in the activity of the predicted protein kinase between the two compared groups, with effect size (values) and direction (>0=activation; <0=inhibition). The color of the bars represents the specificity score (darker the color, higher the specificity). Values of the specificity score >0.9 were considered as statistically relevant. First graph from left: comparison of bFGF stimulated siCTRL cells vs. untreated (UT); all other graphs: comparison of the indicated siTarget vs. siCTRL in the presence of bFGF. *n*=3. **(b-e)** Phylogenetic kinome trees illustrating the family distribution of the upstream kinases predicted to phosphorylate the serine/threonine (STK) and protein tyrosine (PTK) consensus peptides of the PamChip®. Colored dots highlight the kinases where predicted activity is significantly different in the indicated siTarget (**b**: siMAP4K4; **c**: siSTRN4; **d**: siSTRN3, **e**: siSTRIP1) compared to siCTRL in bFGF stimulated conditions in DAOY cells. The coloring scale is based on the mean kinase statistic and ranges from −2 (strong decrease of kinase activity in siTarget vs. siCTRL, red color) to +2 (strong increase of kinase activity in siTarget vs. siCTRL, green color). The size of the circle represents the specificity score.

Figure S8: Volcano plots of peptide chip arrays. (a, b) Individual volcano plots representing the changes in phosphorylation of phospho-serine/threonine (STK, **a**) and phospho-tyrosine (PTK, **b**) peptides in the indicated siTarget versus siCTRL in untreated (UT, top) or bFGF (lower) stimulated conditions in DAOY cells. The peptides that showed significant changes in phosphorylation are highlighted in red ($p < 0.05$) or orange ($p < 0.1$). The p-values were calculated versus siCTRL by ANOVA and post-hoc Dunnett's test in the BioNavigator software. *n*=3.

Figure S1


a
Interacting proteins
BioID2
Linker
Bait
MAP4K4
Addition of biotin
10 nm
+ ATP
Streptavidin IP
Biotinylated proteins
Non-biotinylated proteins
Trypsin digestion
m/z
Mass spectrometry
Linker (13x GGGGS repeats)
CMV promoter
3x FLAG
BioID2
P2A
PuroR
BioID2-CTRL
N-BioID2-M4K4
C-BioID2-M4K4


b
BioID2
Streptavidin
Merged
N-BioID2-M4K4
C-BioID2-M4K4
BioID2-CTRL
DAOY WT
- biotin
+ biotin


c
N-BioID2-M4K4 vs. BioID2-CTRL
-Log10 (p-value)
Log2 (Fold change)
STRN
MAP4K4
TNIK
STRN4
MINK1
CAVIN1
STRIP1
STRN3
MOB4
PP2A-C
p Value=0.05
C-BioID2-M4K4 vs. BioID2-CTRL
CAVIN3
CAV1
PP2A-A

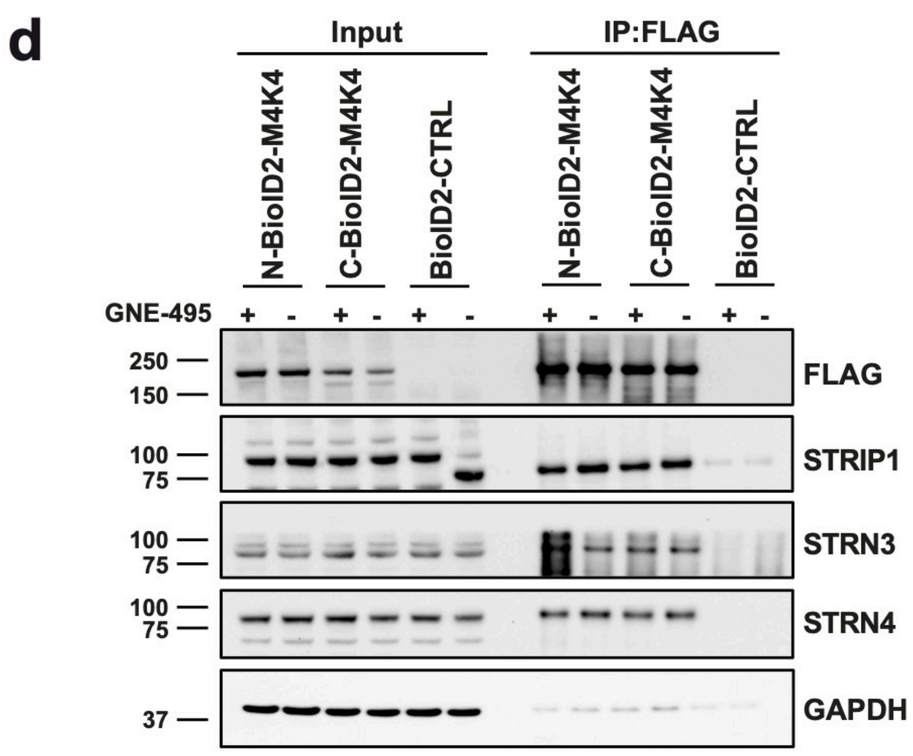

d
Input
IP:FLAG
N-BioID2-M4K4
C-BioID2-M4K4
BioID2-CTRL
GNE-495
250
150
100
75
37
FLAG
STRIP1
STRN3
STRN4
GAPDH

Figure S2

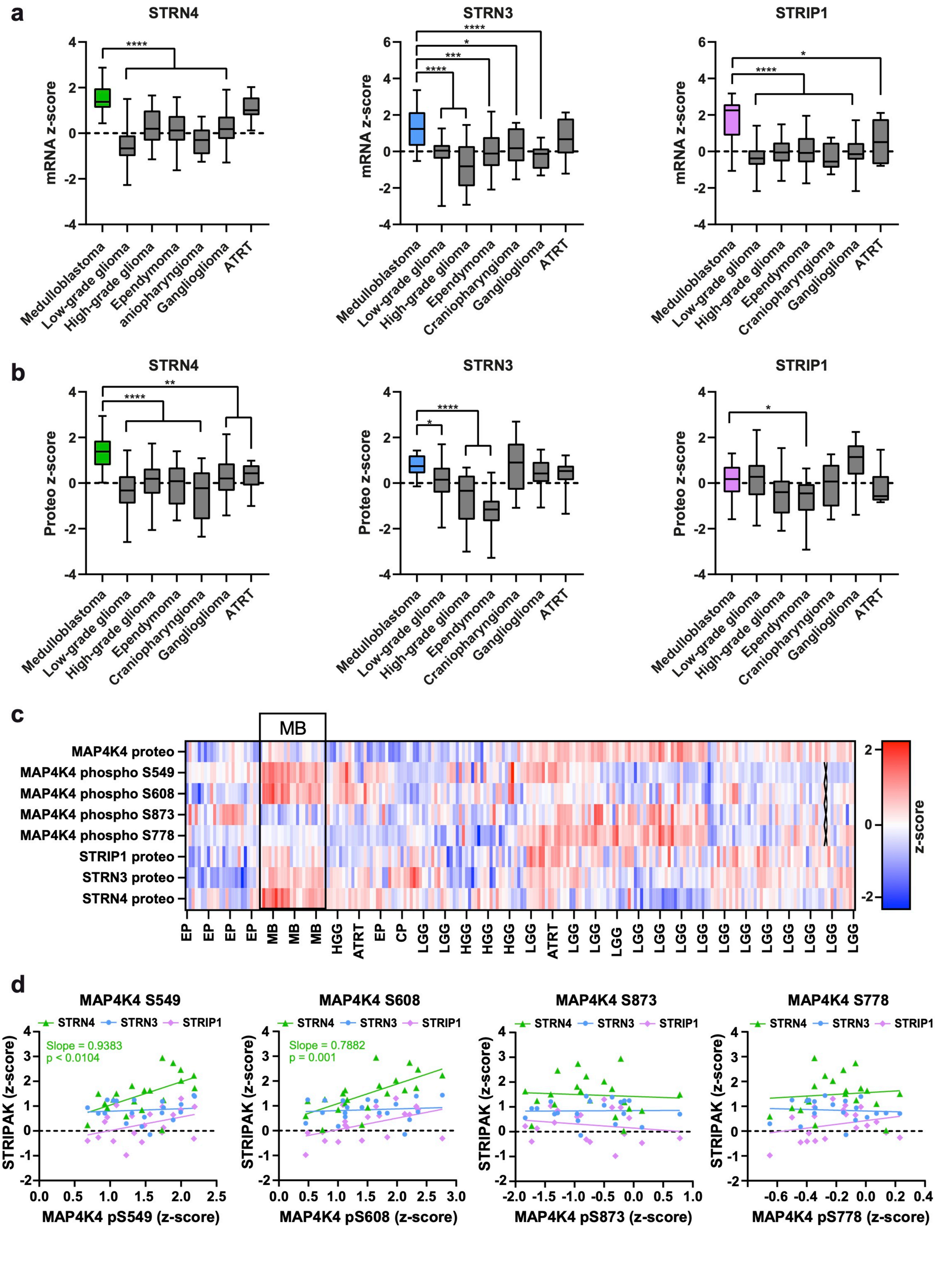
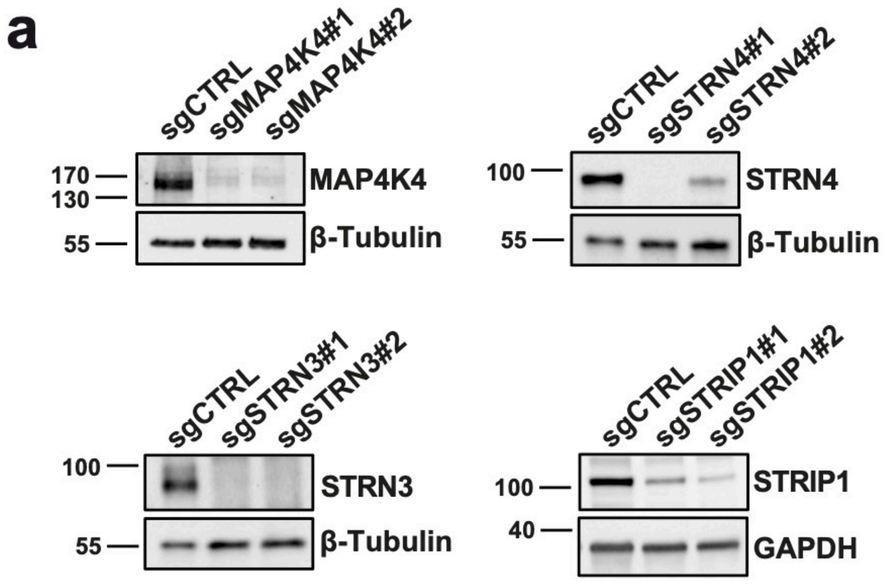
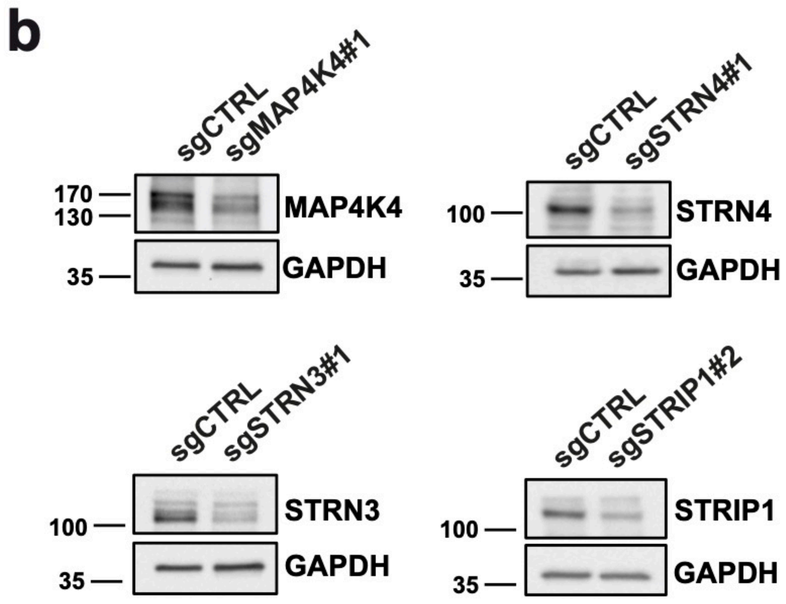
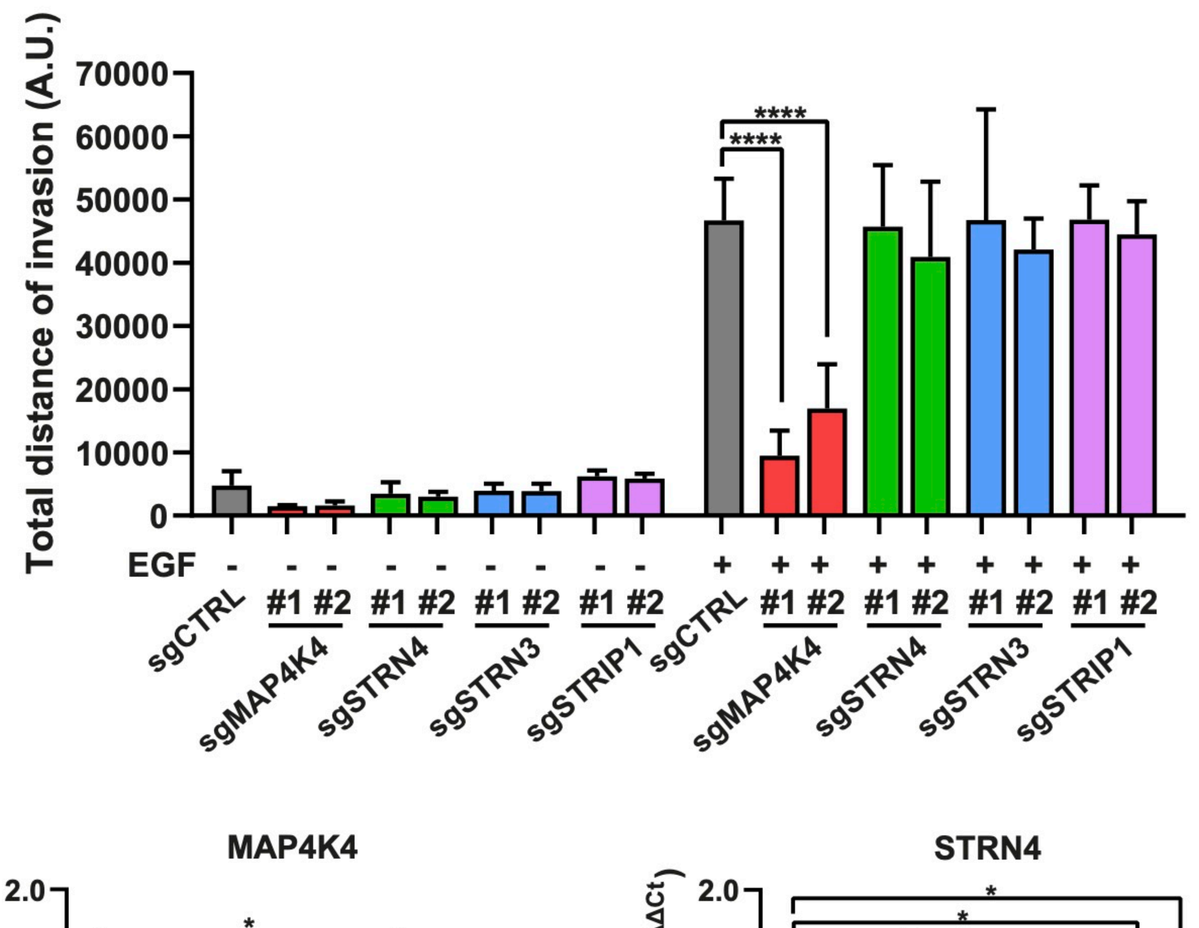

a
STRN4
STRN3
STRIP1
mRNA z-score
Medulloblastoma
Low-grade glioma
High-grade glioma
Ependymoma
aniopharyngioma
Gangliooglioma
Craniopharyngioma
Ganglioglioma
ATRT
b
Proteo z-score
c
MB
MAP4K4 proteo
MAP4K4 phospho S549
MAP4K4 phospho S608
MAP4K4 phospho S873
MAP4K4 phospho S778
STRIP1 proteo
STRN3 proteo
STRN4 proteo
z-score
EP
CP
HGG
LGG
d
MAP4K4 S549
MAP4K4 S608
MAP4K4 S873
MAP4K4 S778
Slope = 0.9383
p < 0.0104
Slope = 0.7882
p = 0.001
STRIPAK (z-score)
MAP4K4 pS549 (z-score)
MAP4K4 pS608 (z-score)
MAP4K4 pS873 (z-score)
MAP4K4 pS778 (z-score)

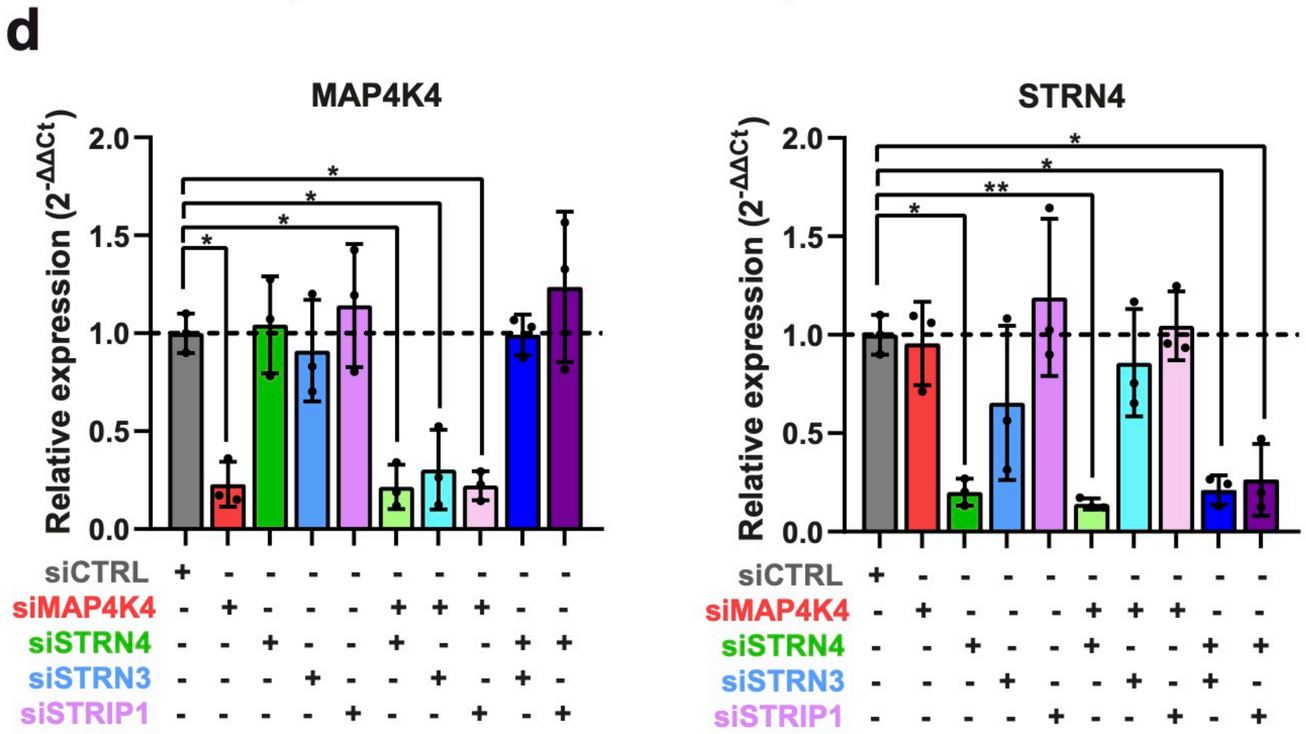
Figure S3

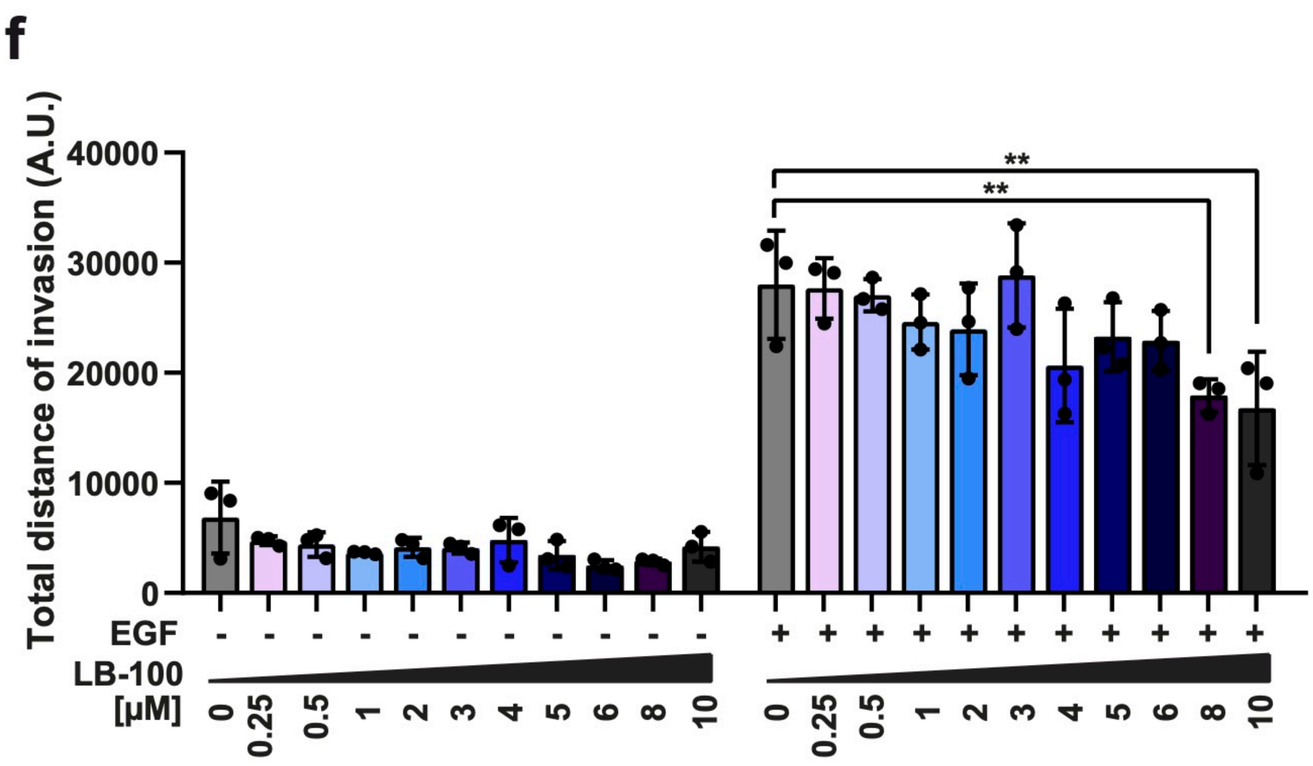

a
sgCTRL
sgMAP4K4#1
sgMAP4K4#2
170
130
MAP4K4
55
β-Tubulin
sgCTRL
sgSTRN4#1
sgSTRN4#2
100
STRN4
55
β-Tubulin
sgCTRL
sgSTRN3#1
sgSTRN3#2
100
STRN3
55
β-Tubulin
sgCTRL
sgSTRIP1#1
sgSTRIP1#2
100
STRIP1
40
GAPDH


b
sgCTRL
sgMAP4K4#1
170
130
MAP4K4
35
GAPDH
sgCTRL
sgSTRN4#1
100
STRN4
35
GAPDH
sgCTRL
sgSTRN3#1
100
STRN3
35
GAPDH
sgCTRL
sgSTRIP1#2
100
STRIP1
35
GAPDH

e
siCTRL + - - - + - - -
siSTRN4 - + - - - + - -
siSTRN3 - - + - - - + -
siSTRIP1 - - - + - - - +
siMAP4K4 - - - - + + + +
170
MAP4K4
100
STRN4
100
STRN3
100
STRIP1
55
β-Tubulin


c
Total distance of invasion (A.U.)
70000
60000
50000
40000
30000
20000
10000
0
****
****
EGF - - - - - - - - - + + + + + + + + +
sgCTRL #1 #2 #1 #2 #1 #2 #1 #2 sgCTRL #1 #2 #1 #2 #1 #2 #1 #2
sgMAP4K4
sgSTRN4
sgSTRN3
sgSTRIP1


d
MAP4K4
STRN4
STRN3
STRIP1
Relative expression (2-ΔΔCt)
2.0
1.5
1.0
0.5
0.0
siCTRL
siMAP4K4
siSTRN4
siSTRN3
siSTRIP1


f
Total distance of invasion (A.U.)
40000
30000
20000
10000
0
**
**
EGF - - - - - - - - - - - + + + + + + + + + + +
LB-100 [μM]
0 0.25 0.5 1 2 3 4 5 6 8 10 0 0.25 0.5 1 2 3 4 5 6 8 10

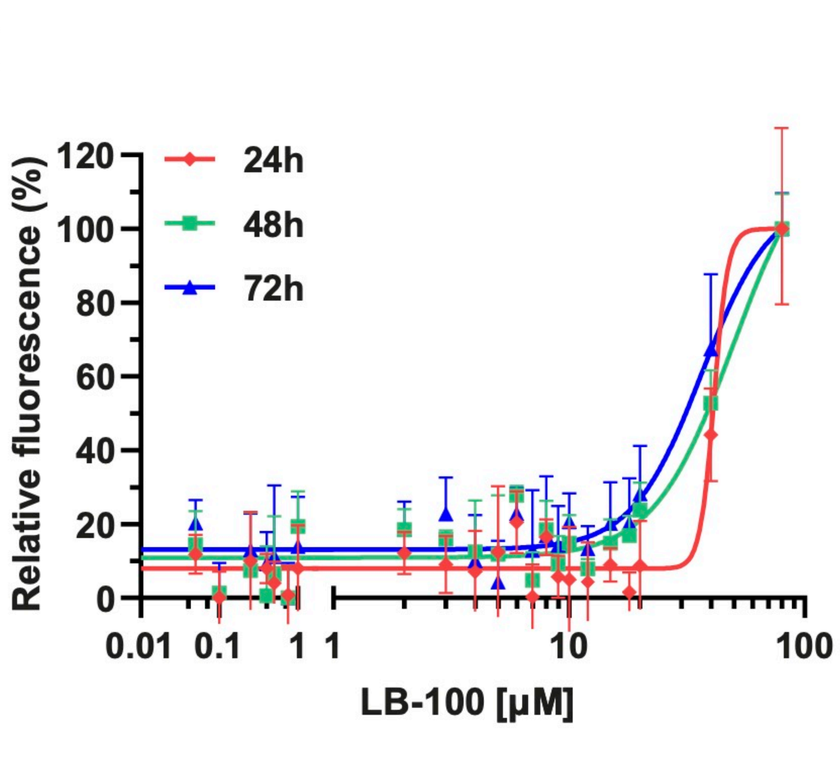

g
Relative fluorescence (%)
120
100
80
60
40
20
0
24h
48h
72h
0.01 0.1 1 1 10 100
LB-100 [μM]

Figure S4

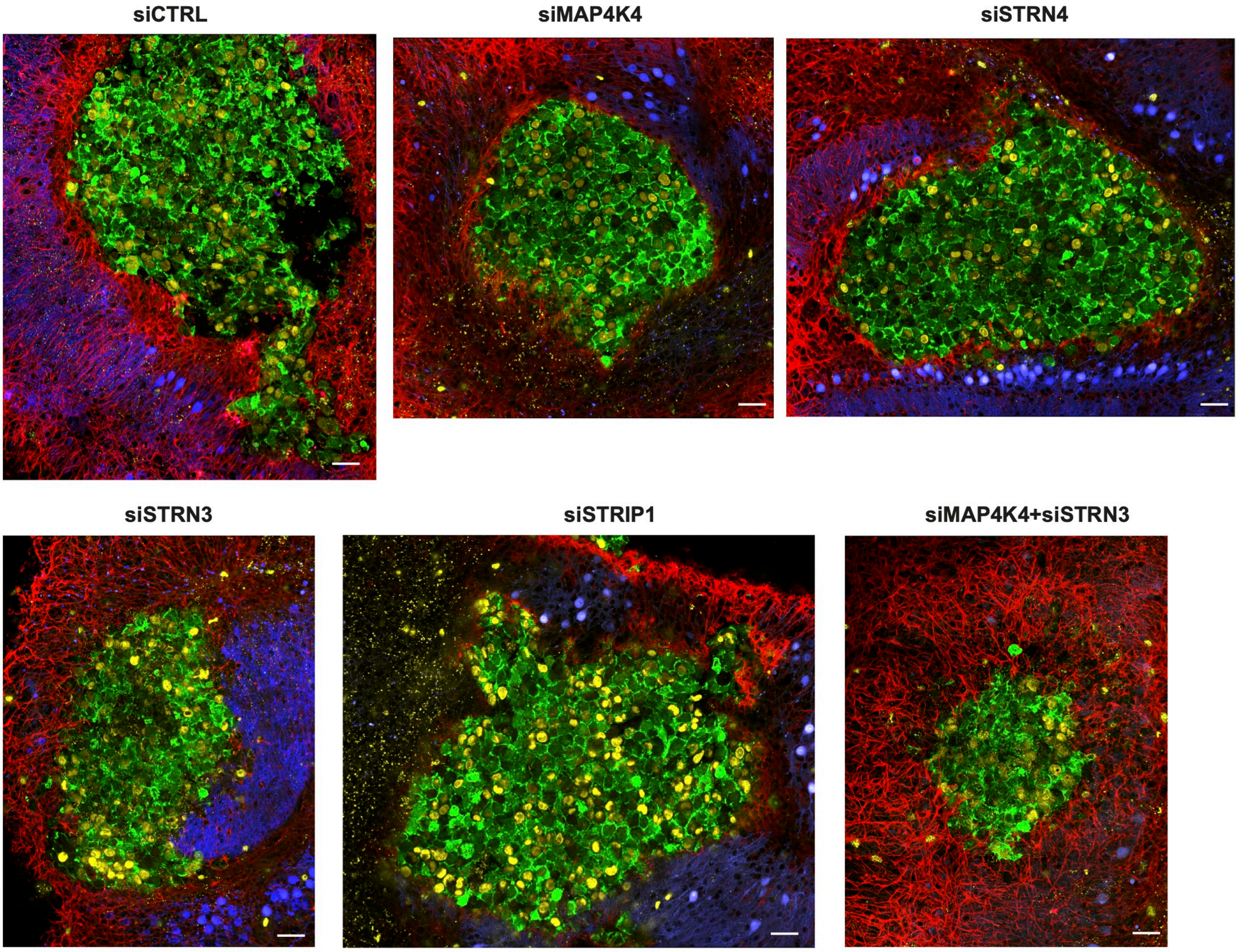
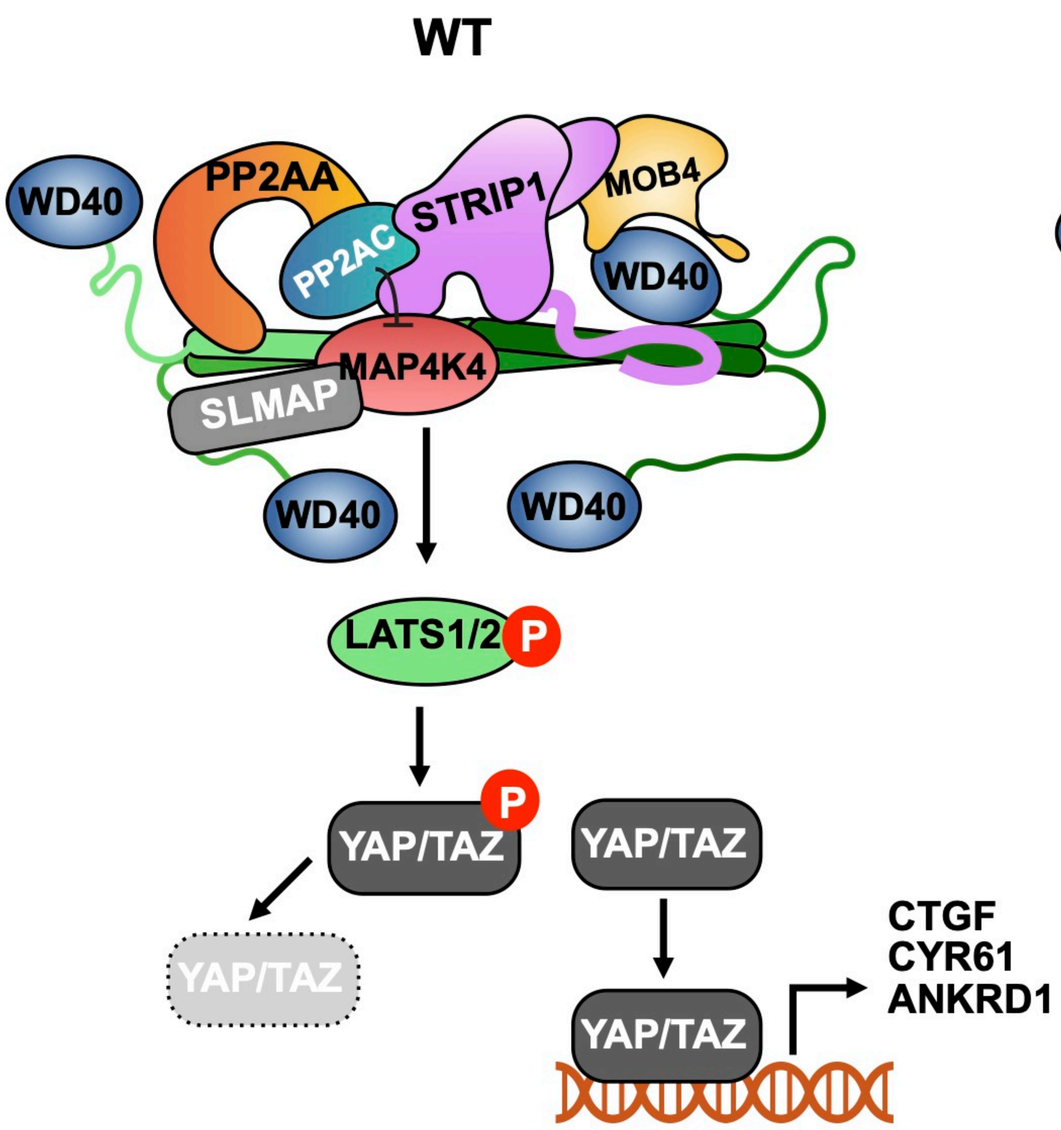

siCTRL
siMAP4K4
siSTRN4
siSTRN3
siSTRIP1
siMAP4K4+siSTRN3

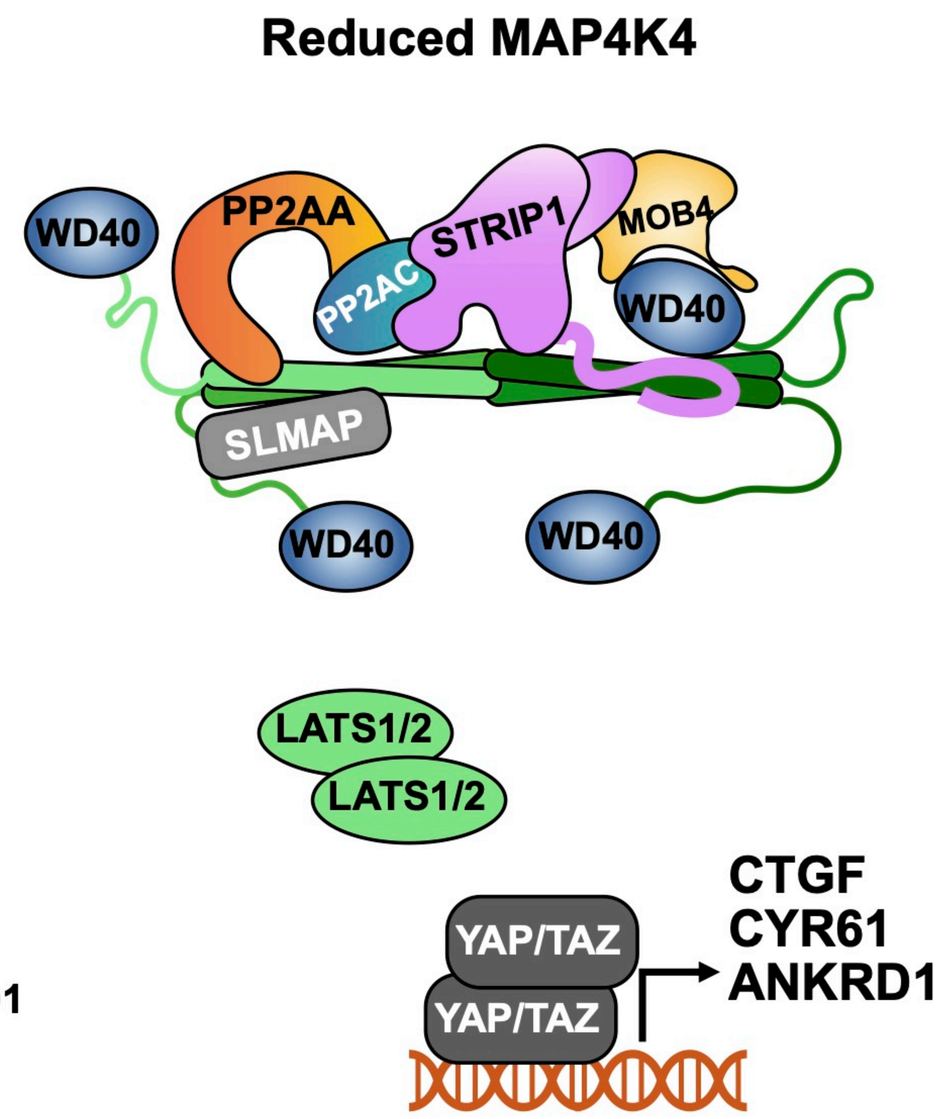
Figure S5

sgCTRL
200 μm
200 μm
200 μm
50 μm
30 μm
sgMAP4K4
200 μm
200 μm
200 μm
sgSTRN3
200 μm
200 μm
200 μm
sgSTRIP1
200 μm
200 μm
200 μm

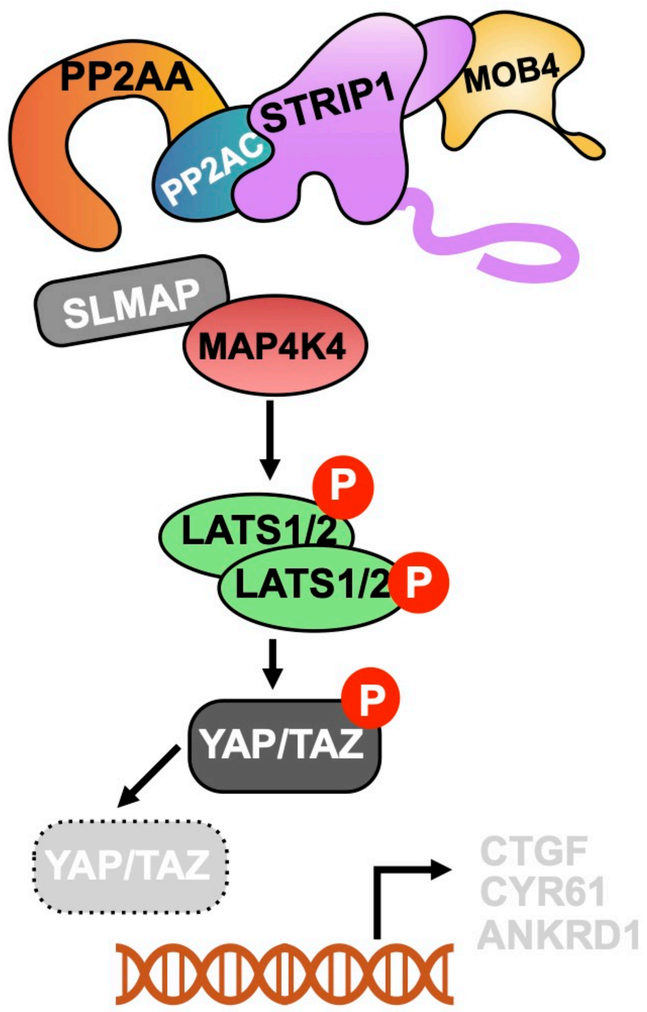
Figure S6



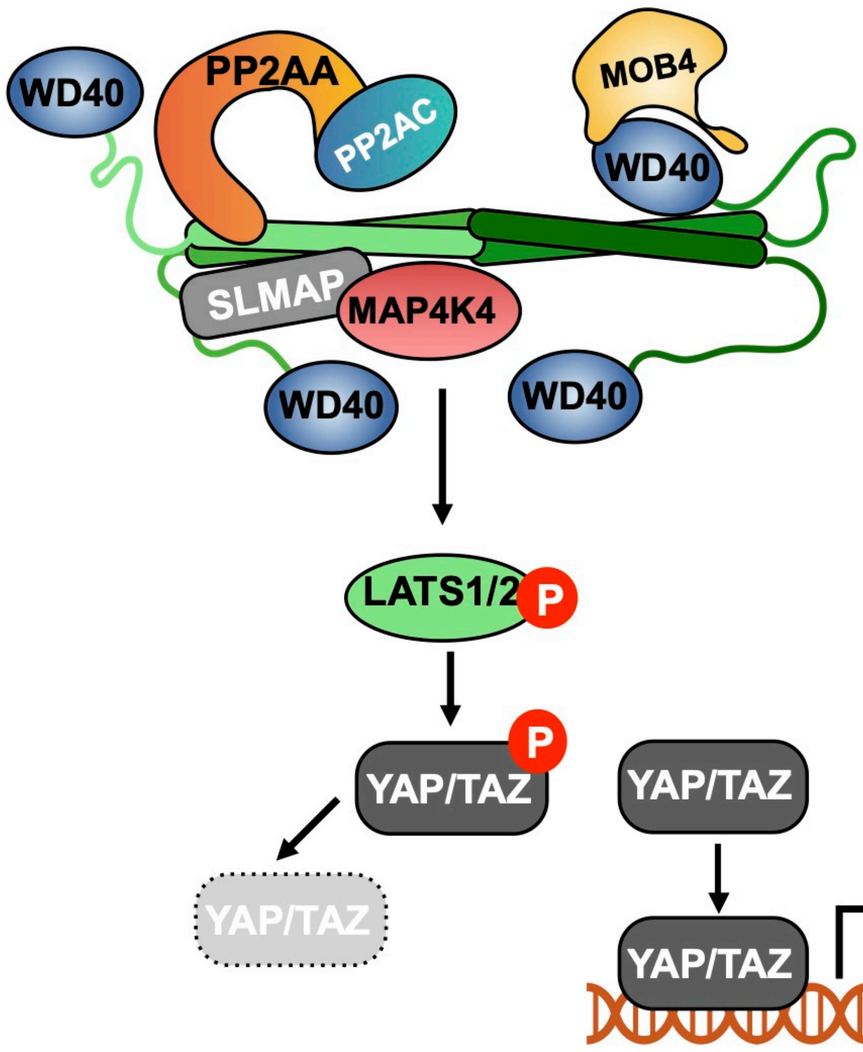
Normal growth



Increased growth



Reduced growth



Normal growth

Figure S7

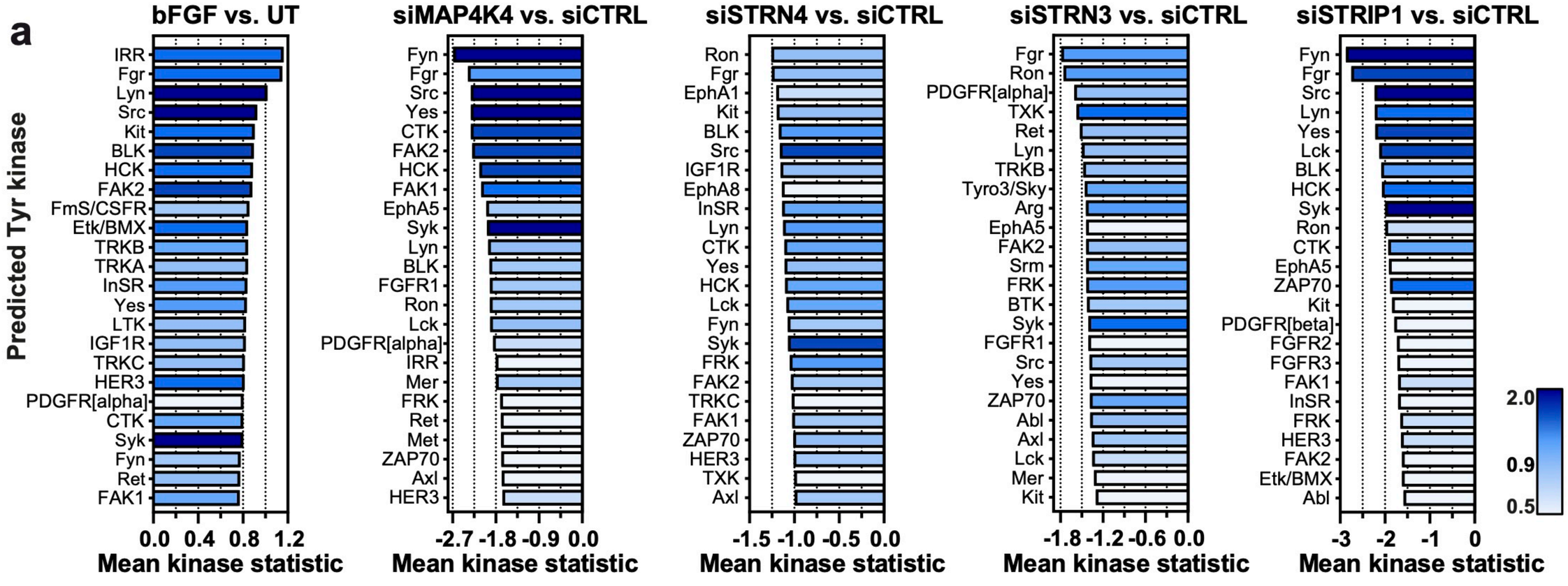
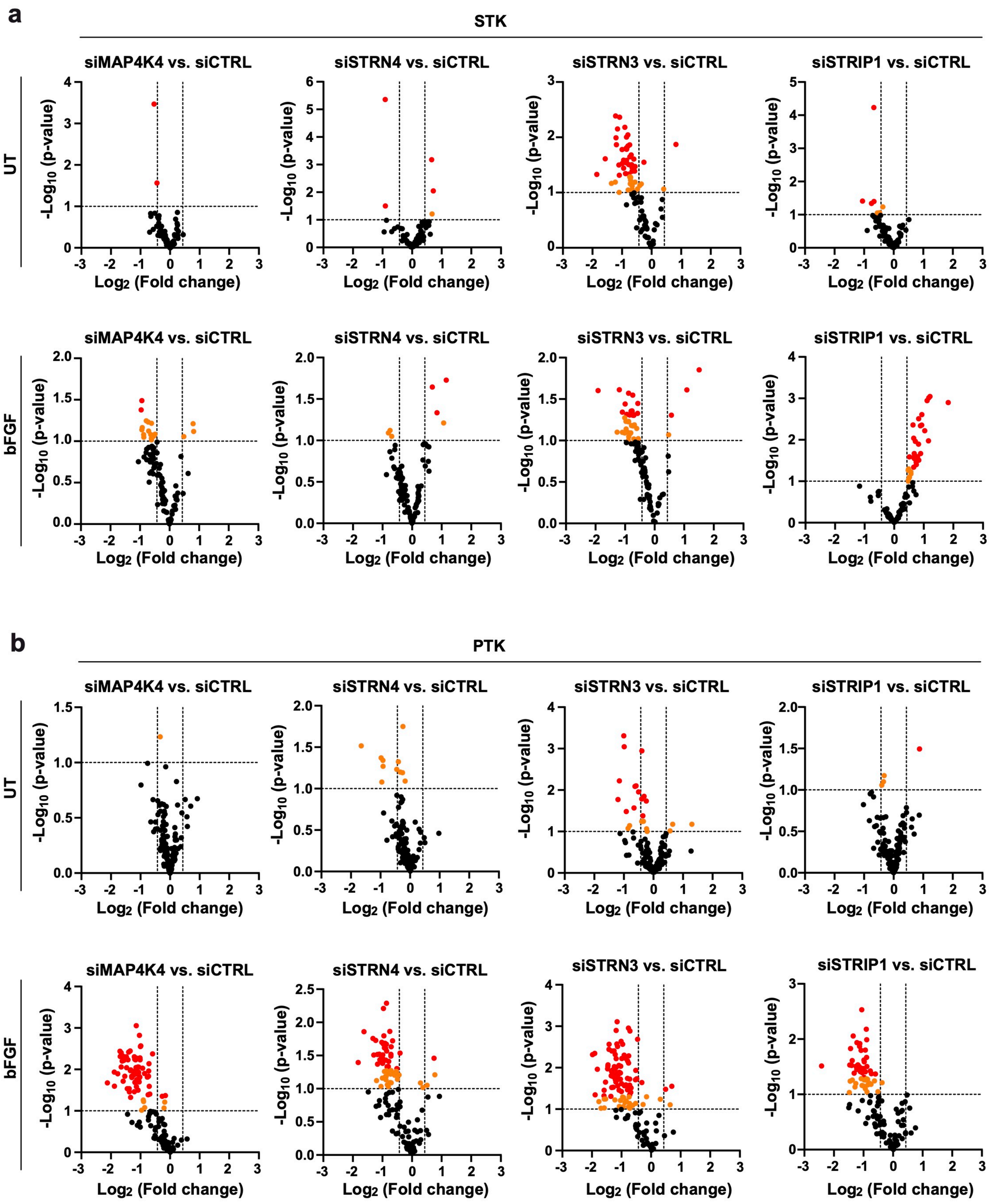

a
bFGF vs. UT
siMAP4K4 vs. siCTRL
siSTRN4 vs. siCTRL
siSTRN3 vs. siCTRL
siSTRIP1 vs. siCTRL
Predicted Tyr kinase
Mean kinase statistic
b
c
d
e
TK
TKL
STE
CMGC
CK1
AGC
CAMK
Kinase statistic
-2 0 2
Specificity Score
0.5 2
2.0
0.9
0.5

Figure S8


a
STK
UT
bFGF
siMAP4K4 vs. siCTRL
siSTRN4 vs. siCTRL
siSTRN3 vs. siCTRL
siSTRIP1 vs. siCTRL
-Log10 (p-value)
Log2 (Fold change)
b
PTK